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Silk gene transcripts in the developing tubuliform glands of the Western black widow, *Latrodectus hesperus*

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Abstract. Spiders spin a variety of task-specific silk fibers, each composed of one or more unique proteins synthesized within specialized glands in the spider's abdomen. Tubuliform glands are the source of the large diameter silk fibers used by many species in the construction of egg cases. Unlike other silk glands that synthesize protein throughout a spider's lifetime, the tubuliform glands synthesize silk in association with the maturation of oocytes, culminating in the production of an egg case. In the Western black widow, *Lactrodectus hesperus* Chamberlin & Ivie (1935), egg case fibers are composed of at least three proteins: tubuliform spidroin 1 (TuSp1), egg case protein-1 (ECP-1), and egg case protein-2 (ECP-2). Here, we present the first study to quantify the pattern of transcription for these three genes in a developmental series of tubuliform glands from *L. hesperus*. All three transcripts increase in abundance prior to the production of an egg case, but at different time points. After egg case production, silk transcripts are still detectable in the tubuliform glands. Relative abundance of TuSp1 mRNA is several orders of magnitude higher than that of ECP-1 and ECP-2 at almost every stage. The relative abundance of silk transcripts across the reproductive life history of black widows suggests differential regulation of silk gene transcription within tubuliform glands.

Keywords: Spider silk, egg case, tubuliform spidroin 1, egg case protein-1, egg case protein-2

Orb-weaving spiders and their relatives (Orbiculariae) possess a set of seven distinct gland types that produce silks used for functions such as prey capture and swathing, habitat construction, and reproduction. Each gland-specific fiber is made up of one or more unique structural spider silk proteins (spidroins, contraction of "spider fibroins") (Guerette et al. 1996). Tubuliform spidroin 1 (TuSp1) is the major protein component of the large diameter silks composing the egg case that forms the protective environment for the developing spiderlings (Garb & Hayashi 2005; Tian & Lewis 2005; Hu et al. 2005a; Zhao et al. 2005; Huang et al. 2006). Large diameter silk fibers are the product of tubuliform (cylindrical) glands (Stubbs et al. 1992; Moon 2003; Vasanthavada et al. 2007). Two additional silk associated proteins, egg case proteins-1 and -2 (ECP-1 and ECP-2), have been identified in the tubuliform glands of black widows (Hu et al. 2005a, 2005b).

In contrast to most silk glands that synthesize protein throughout the lifetime of a spider, tubuliform glands are found only in mature females and their development parallels the maturation of the ovaries such that the glands reach a maximal size in gravid spiders (Kovoor 1990; Moon 2003). This unique feature of egg case silk synthesis makes the tubuliform gland a potential model system for investigating the regulatory mechanisms responsible for dramatically increasing spider silk gene expression. Histochemical and morphological observations of tubuliform glands indicate that protein synthesis peaks just prior to the construction of an egg case (Candelas et al. 1986; Moon 2003; Huang et al. 2006). However, the signaling pathways and regulatory molecules used to influence egg case silk protein production are unknown. With the advent of highly sensitive nucleic acid techniques such as quantitative PCR, we can begin to address questions about the transcriptional regulation of spider silk.

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Here, we use quantitative PCR to examine the transcript levels for the three tubuliform gland-specific silk genes over a developmental time series for the Western black widow, *Latrodectus hesperus* (Araneae: Theridiidae). Our goal is to correlate vitellogenesis with the development of tubuliform glands and the relative levels of egg case-specific gene transcripts. Our results provide a foundation for gene expression studies of spider silk glands both within and across gland types and species. More broadly, we show the utility of black widow tubuliform glands as a model system for the investigation of signal pathways responsible for the transcriptional regulation of silk proteins.

METHODS

Spider collection, dissection, and staging.—Female Western black widow spiders (*L. hesperus*) were collected in Riverside and Fullerton, California (USA). Voucher specimens have been deposited in the Entomology Research Museum at the University of California, Riverside (UCRC ENT 229278). Spiders were housed in individual containers and maintained at an ambient temperature of 27° C with a 14:10 h day:night cycle. We fed the spiders mealworms (juvenile *Tenebrio molitor*) every three weeks. Tubuliform glands and ovaries/eggs were isolated from individual spiders by dissection under 0.15 M sodium chloride, 0.015 M sodium citrate buffer (SSC). We immediately froze the glands in liquid nitrogen and then stored them at −80° C. Individual spiders were assigned to one of seven life history stages based in part on the scheme developed by Trabalon et al. (1992). Virgin spiders (V) were identified as spiders that had undergone at least one molt in captivity and possessed small, undeveloped oocytes. Gravid spiders (G) possessed a loose mass of large yolky eggs within their abdomens. Using the criteria established by Trabalon et al. (1992), black widow spiders were characterized as being in either early (Vitel-E) or late vitellogenesis (Vitel-L) based on measurements of the overall diameter of the oocytes and the

size and distribution of the yolk granules within those oocytes. The final three stages represent time points following the production of an egg case: within 8 h of spinning (D0), 24 h after (D1), or 48h after (D2).

Microscopy.—Ovaries, eggs, or tubuliform glands were prepared for microscopy by an overnight fixation in 2% formaldehyde, 2% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4) at 4° C. We then rinsed the tissue in 0.1 M sodium phosphate buffer and stored it under buffer at 4° C. Morphological characteristics of ovaries and eggs were visualized using an environmental scanning electron microscope (Hitachi TM1000). We photographed tubuliform glands using a Leica MZ125 stereomicroseope with a digital camera (Spot Insight). We determined the ooctye and gland diameters using the measurement tools available from the image-capture software of each respective microscope. We calculated mean gland diameter from measurements of multiple tubuliform glands from three individuals at each life history stage. In order to test the categorization of oocytes and mean gland diameter across various reproductive life history stages, we used one-way ANOVA assuming unequal variance. We used SPSS software (SPSS Inc., Chicago, Illinois) to conduct the statistical analysis.

Total RNA isolation and cDNA synthesis.—Total RNA was extracted from tubuliform gland tissue by homogenization in TRIzol® Reagent (Invitrogen). We processed glands from individual spiders separately with the exception of the virgin spiders in which glands from multiple individuals were pooled in order to obtain sufficient quantities of total RNA for cDNA synthesis. Genomic DNA carryover was eliminated with TURBO^{TM}-DNase (Ambion). The absence of any product in standard PCR amplification of every total RNA sample used in this study with the TuSp1 primer set (see below) supports the conclusion that the total RNA samples were free of genomic DNA contamination. We synthesized single-stranded cDNA from 1 µg of total RNA with Superscript® III reverse transcriptase (Invitrogen) primed from an anchored oligo(dT) primer.

Real-time quantitative PCR.—Tubuliform silk gland cDNA formed the template for real-time quantitative PCR (RT-qPCR) amplification. We designed primers to amplify the C-terminal encoding region of *L. hesperus TuSp1* (forward primer LhTuSp1_3733F: CCTGGTTTGATTGTAGGACCCTC; reverse primer LhTuSp1_3993R: GGATTTCCGCTTTGAA TGGATG). Primers used for the amplification of transcript for the egg case proteins ECP-1 and ECP-2 in *L. hesperus* were based on those of Hu et al. (2006). Calreticulin, a calcium-binding protein of the endoplasmic reticulum, was used as the housekeeping reference gene for the RT-qPCR reactions. We designed primers for calreticulin from the *L. hesperus* calreticulin sequence (GenBank accession number GQ402146; forward primer Cal Lh620F, AGAAAATGAAAGATCCCGAGGC; reverse primer Cal Lh801R, AATTTGAGGTGGTTCCC ACTCTC). We confirmed specificity of the primer sets through sequencing of the respective PCR products at every developmental stage.

RT-qPCR was completed using the MyIQ5 thermocycler (BioRad) and associated software (Version 2.0). Each 20 µl reaction volume contained 0.1 µg of template cDNA and 200 nM of each primer in SYBR Green Supermix (BioRad). The reaction profile included 40-cycles of 15 s denaturation at 95° C, 40 s annealing at 60° C, and 45 s extension at 72° C followed by a 56° C to 94° C melt curve analysis. Reactions using cDNAs from individual spiders representing each of the reproductive life history stages (or pooled cDNAs in the case of virgin spiders) were done in triplicate with four independent trials for each stage. Efficiency of each primer set was determined using a ten-fold serial dilution of G stage cDNA. The reference gene, calreticulin, was found to have an efficiency of 90% while each of the silk primer sets had an efficiency of greater than 100%. We confirmed the absence of primer:dimer formation during PCR by using a melt curve analysis. Abundance of transcript levels was calculated relative to the reference gene (calreticulin) and a control condition (D0 stage) using the method developed by Pfaffl (2001). We defined the D0 stage as the control condition based on the finding that the cycle threshold (C_t) values at which the target-specific signal exceeds background for all transcripts tested supported the assumption that mRNA levels were lowest immediately following egg case production.

RESULTS AND DISCUSSION

Oocyte and gland morphology change relative to vitellogenesis.—The process of vitellogenesis alters the size and surface morphology of the developing oocytes as visualized by scanning electron microscopy (Fig. 1). Stages of oocyte development similar to those noted by Trabalon et al. (1992) for two agelenid species were identified in the black widow. We found each of the stages to be distinct based on measurements of oocyte diameter [$F_{(7,454)} = 1403.6$, $P < 0.001$]. Oocytes increase in mean diameter from 70 ± 1 µm (S.E.) in virgin spiders (Fig. 1A) to 183 ± 5 µm in early vitellogenic (Fig. 1C) and 295 ± 4 µm late vitellogenic (Fig. 1E) spiders. These developing oocytes attach to the ovarian wall by means of a pediele (Figs. 1C & E), but are released into the abdomen of the gravid spider (Fig. 1G). The oocytes in the gravid female have a mean diameter of 574 ± 17 µm. In addition to size, the accumulation and organization of yolk granules within the oocytes are distinguishing characteristics for the stages of vitellogenesis (Figs. 1A, C, E and G).

Similarly, we also found the appearance of the tubuliform glands to differ significantly with the reproductive stage of the spider [$F_{(6,160)} = 59.82$, $P < 0.001$] (Fig. 1). The tubuliform glands of virgin spiders (Fig. 1B) had the smallest mean diameter (125 ± 2 µm) relative to the glands from any of the other stages. Tubuliform gland diameter increased from early (Fig. 1D) to late vitellogenesis (Fig. 1F), reaching a maximum mean diameter of 340 ± 8 µm in gravid spiders (Fig. 1H). Following the production of an egg case, the tubuliform glands had a flattened appearance and returned to a mean diameter of 157 ± 7 µm (data not shown).

Silk gene transcript levels in tubuliform glands vary with reproductive life history stage.—RT-qPCR detected TuSp1, ECP-1 and ECP-2 silk transcripts within the tubuliform glands at all reproductive life history stages examined, including virgin spiders (Fig. 2). The relative quantities of all three silk transcripts (TuSp1, ECP-1, and ECP-2) reached their highest level during late vitellogenesis (Vitel-L). TuSp1 and ECP-2 mRNA levels increased during early vitellogenesis while

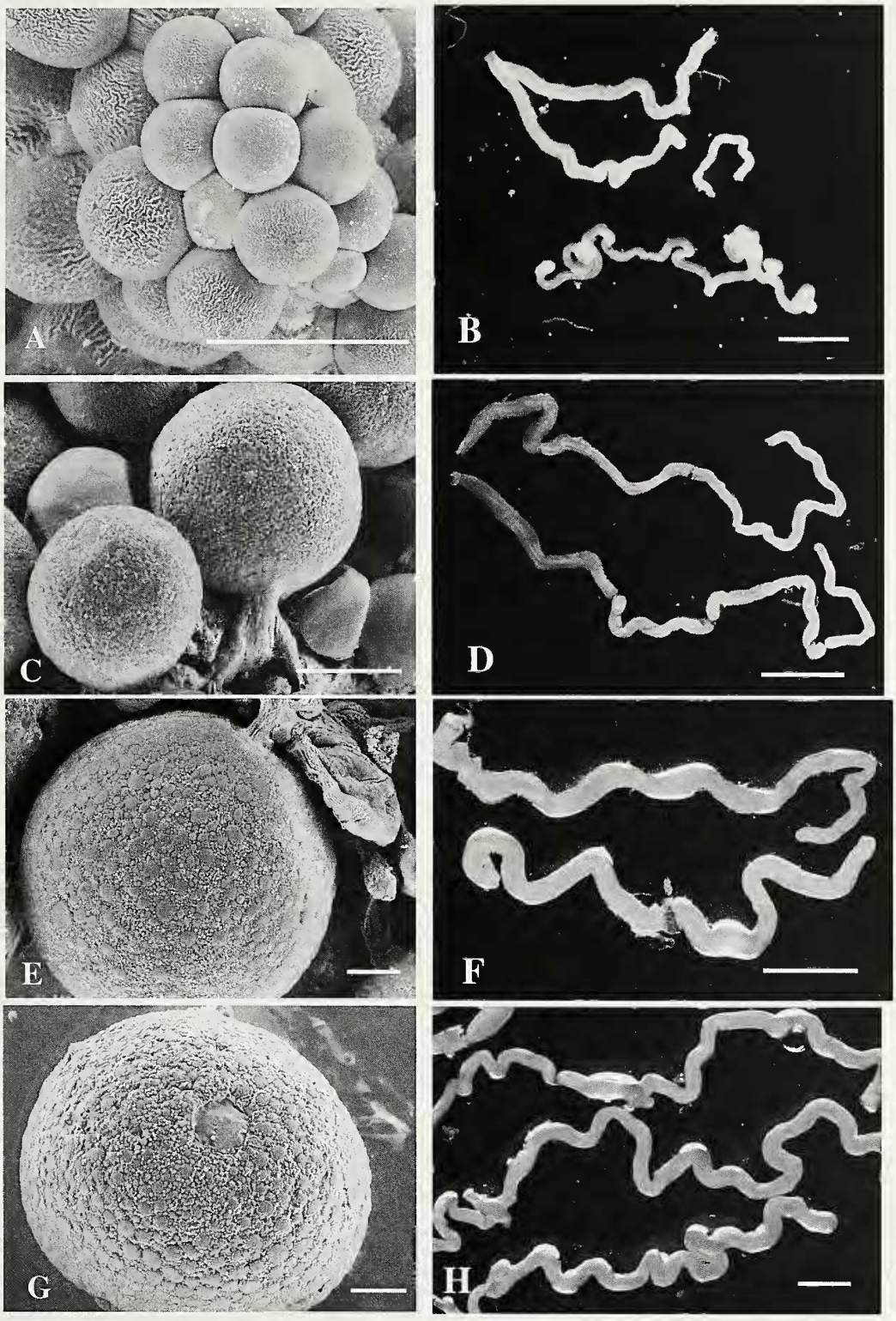

Figure 1.—Tubuliform gland morphology changes coincident with vitellogenesis in *L. hesperus*. (A & B) Oocytes and tubuliform glands in virgin spiders are small (mean diameters; 70 ± 1 μm S.E. and 125 ± 2 μm S.E., respectively). (C & D) During early vitellogenesis, the oocytes and glands begin to increase in size (mean diameters; 183 ± 5 μm S.E. and 174 ± 8 μm, respectively). (E & F) Growth of oocytes and tubuliform glands continue into late vitellogenesis (mean diameters; 295 ± 4 μm S.E. and 189 ± 8 μm, respectively) reaching a maximum (mean diameters; 574 ± 17 μm S.E. and 340 ± 8 μm, respectively) in gravid spiders (G & H). Scale bars for all oocytes images = 100 μm. Scale bars for all gland images = 1mm.

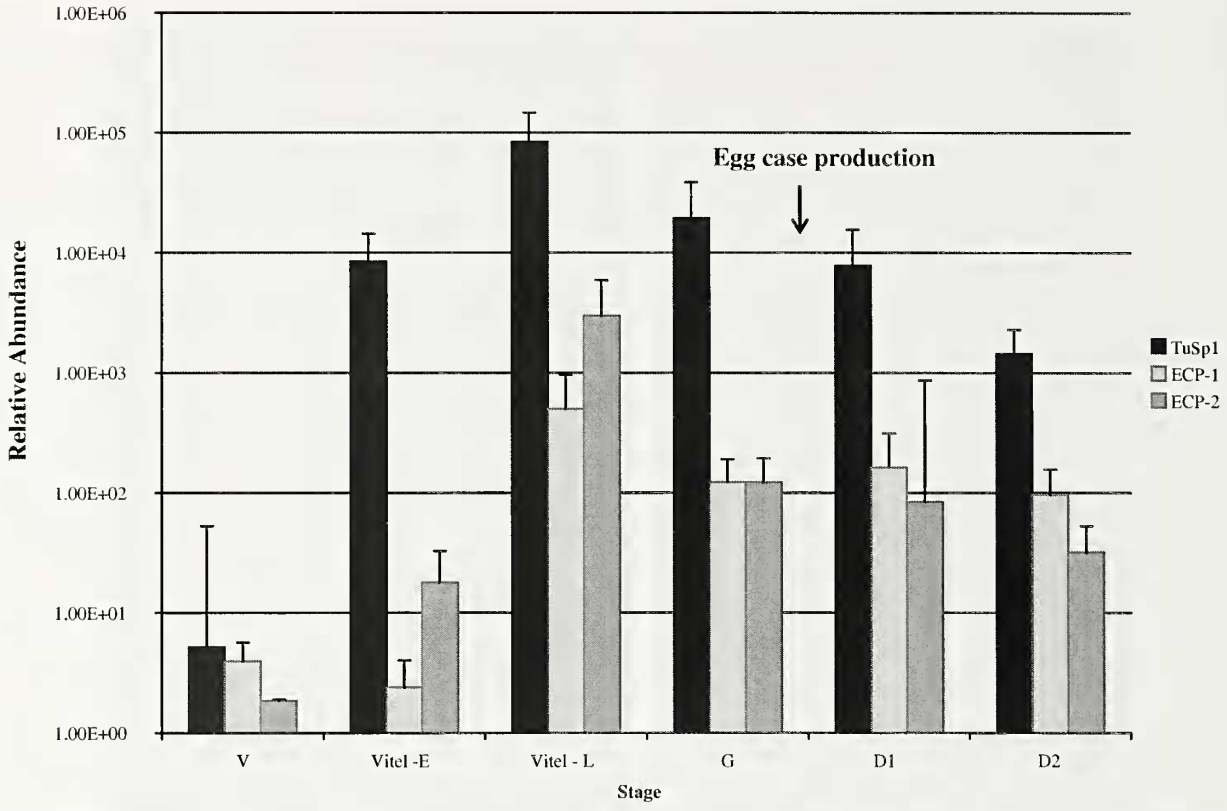

Figure 2.—Silk gene transcript levels vary with reproductive life history stage for the tubuliform gland of *L. hesperus*. The relative abundance of the tubuliform silk mRNAs, TuSp1, ECP-1, and ECP-2 was determined by RT-qPCR. Transcripts for all three silks were detected in tubuliform glands from virgin spiders. The quantity of silk mRNAs present in the gland increases through vitellogenesis (Vitel-E and Vitel-L), and is maintained following the production of an egg case. Levels of TuSp1 mRNA exceed those of the ECPs at all stages from early vitellogenesis on. All samples were tested in triplicate and normalized to the housekeeping gene, calreticulin. The D0 stage was used as the control condition. Results presented here are the average of four independent trials. Error bars indicate SEM.

ECP-1 mRNA levels did not increase until late vitellogenesis. The amount of each of the silk transcripts present in the glands at 48 h post egg case production was similar to that in gravid spiders. It has yet to be determined whether the accumulation of silk mRNAs over these developmental stages is due to increased initiation of transcription, changes in mRNA stability, or some combination of the two.

Given the specific function of the tubuliform gland in spider reproduction, it has been proposed that egg case silks are likely under hormonal control (Kovoor 1990). Hormonal regulation of silk production has been demonstrated in the silkworm moth, *Bombyx mori* (Fukuta et al. 1993; Xu et al. 1994; Tang et al. 2007). Juvenile hormone and ecdysterol have been shown to influence the expression of the lepidopteran silk protein, seroin (Žurovec et al. 1998), and it is known that these same hormones influence the process of vitellogenesis in spiders (Trabalon 1992; Pourie & Trabalon 2003).

While a hormone might initiate a signaling pathway leading to silk gene expression, a transcription factor is ultimately required to promote the binding of RNA polymerase to the promoter region of the silk gene. One candidate protein has been identified in the black widow spider (Kohler et al. 2005). Silk gland subset factor (SGSF) is a class II basic helix-loop-helix (bHLH) transcription factor expressed primarily in silk glands. The expression level of SGSF is reported to be elevated in late vitellogenesis, coincident with the peak of silk transcription demonstrated here. Kohler et al. (2005) have proposed that SGSF may function in concert with other regulatory proteins to trigger the differentiation of the tubuliform gland itself, as well as to control the expression of the egg case silk genes.

The relative amount of TuSp1 mRNA present in black widow tubuliform glands was, with the exception of virgin spiders, consistently greater than that of ECP-1 or ECP-2 (Fig. 2). The most dramatic difference in relative abundance occurred during early vitellogenesis when TuSp1 levels were ~3500 fold greater than ECP-1 and ~470 fold greater than ECP-2. At this same stage, ECP-2 was 7.5 fold more abundant than ECP-1. Following the production of an egg case, the differences between the amounts of TuSp1 and ECP message were less extreme. This co-expression of all three genes is consistent with the observation that the large diameter silk fibers of the black widow egg case are a trimeric complex of the three proteins (Hu et al. 2006). Our data are consistent with and expand upon earlier findings that TuSp1 is the major protein product of the black widow tubuliform gland and that the abundance of TuSp1 mRNA exceeds that of the ECPs at all stages (Garb & Hayashi 2005; Hu et al. 2006). It has been proposed that ECP-1 and -2 function as intermolecular linkers within the tubuliform silk fiber through the formation of disulfide bridges (Hu et al. 2006). The difference in transcript levels of ECP-2 compared to ECP-1, however, has implications for the putative role of these proteins in fiber assembly. ECP-2, with its potential for disulfide bond formation at its N-

terminus and beta-pleated sheet formation at its C-terminus, appears to be required in greater abundance and at an earlier stage in silk protein synthesis than ECP-1.

The D0 stage (within 24 h following egg case production) is not represented in the profile (Fig. 2) since the cycle threshold (C_t) values from this population of spiders were used in the calculation of relative abundance (Pfaffl 2001). D0 C_t values (number of thermocycles required to detect PCR product above background) were consistently higher than any other stage for all transcripts, supporting the assumption that mRNA levels are lowest at this stage. It should be noted, therefore, that the relative abundance of mRNA observed at D1 represents a marked increase from the reduced transcript levels found at D0. The levels of TuSp1 and ECP mRNAs detected in the D1 and D2 tubuliform glands are comparable to the abundance found in late vitellogenesis. Further research is required to determine the protein synthetic activity of these post-egg case glands.

Analysis of silk gene transcripts in the tubuliform glands of black widows has revealed that transcript levels are associated with vitellogenesis, but each gene displays a distinct pattern. Given the relative amounts and profile of each silk transcript described in this paper, it seems unlikely that a single transcriptional regulatory mechanism could account for the expression of all three genes. These results support the use of *Latrodectus* tubuliform glands as a powerful model system for further investigation into the details of the regulatory mechanisms of spider silk gene expression. Future comparative studies of silk gene expression across species and gland types will add new dimensions to our understanding of both the mechanistic and evolutionary elements of silk synthesis in the impressive diversity of silk glands in spiders.

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